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Activities of Tobramycin and Six Other Antibiotics against *Pseudomonas aeruginosa* Isolates from Patients with Cystic Fibrosis

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The in vitro activity of tobramycin was compared with those of six other antimicrobial agents against 1,240 *Pseudomonas aeruginosa* isolates collected from 508 patients with cystic fibrosis during pretreatment visits as part of the phase III clinical trials of tobramycin solution for inhalation. The tobramycin MIC at which 50% of isolates are inhibited (MIC_{50}) and MIC_{90} were 1 and 8 μg/ml, respectively. Tobramycin was the most active drug tested and also showed good activity against isolates resistant to multiple antibiotics. The isolates were less frequently resistant to tobramycin (5.4%) than to ceftazidime (11.1%), aztreonam (11.9%), amikacin (13.1%), ticarcillin (16.7%), gentamicin (19.3%), or ciprofloxacin (20.7%). For all antibiotics tested, nonmucoid isolates were more resistant than mucoid isolates. Of 56 isolates for which the tobramycin MIC was ≥16 μg/ml and that were investigated for resistance mechanisms, only 7 (12.5%) were shown to possess known aminoglycoside-modifying enzymes; the remaining were presumably resistant by an incompletely understood mechanism often referred to as "impermeability."

Chronic bacterial pulmonary infections are common in patients with cystic fibrosis (CF). Although other pathogens are seen early in life, as patients reach adolescence, *Pseudomonas aeruginosa* becomes the predominant respiratory pathogen. By age 17 approximately 60% of CF patients in the United States are chronically infected with *P. aeruginosa* in their respiratory tracts (9). In a recent survey almost 50% of Italian CF patients showed colonization with *P. aeruginosa*, with the colonization rate reaching 90% of patients in the 31- to 35-year-old age group (28). The aggressive antibiotic treatment of pulmonary *P. aeruginosa* infections has led to significant improvement in morbidity and mortality from the disease; in the past 20 years the median age of survival has increased from 14 to 30.1 years (9, 11).

Increased exposure to antibiotics raises concern regarding the potential for emergence of resistant *P. aeruginosa* isolates. Investigators have observed a correlation between resistance of *P. aeruginosa* and antibiotic administration (7, 10, 22, 30). In organisms isolated from patients with infections other than cystic fibrosis, aminoglycoside resistance is often caused by modifying enzymes (20, 21). In contrast, *P. aeruginosa* isolates from CF patients commonly exhibit a broad-spectrum aminoglycoside resistance that is caused by an incompletely understood mechanism often referred to as "impermeability" (16, 25). This impermeability resistance has been shown to be independent of known aminoglycoside-modifying enzymes, but no specific mechanism has been defined. The term impermeability is therefore used to differentiate known enzymatic resistance from the undefined broad-spectrum aminoglycoside resistance (16, 20).

The goal of this study was to examine the antibiotic resistance patterns in a large number of geographically diverse *P. aeruginosa* clinical isolates and to evaluate mechanisms of resistance to aminoglycosides in isolates for which tobramycin MICs are high. More than 1,200 *P. aeruginosa* isolates were available from CF patients who enrolled in a multicenter study to assess the safety and efficacy of tobramycin solution for inhalation. We determined the MICs of tobramycin and six other antibiotics and analyzed the susceptibility patterns of the isolates. In addition, the resistance patterns were examined for mucoid and nonmucoid phenotypes of *P. aeruginosa* isolates separately.

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MATERIALS AND METHODS

Culture of sputum. Five hundred eight patients were enrolled in clinical trials in the United States for evaluation of the safety and efficacy of tobramycin solution for inhalation (5, 24). Sputum samples were collected on the first day of a regularly scheduled patient visit prior to the start of treatment (baseline). Samples were collected over a period of 9 months between August 1995 and May 1996. Sputum specimens were shipped on wet ice by overnight carrier to a central laboratory and were cultured within 48 h of collection. Quantitative cultures of sputum were done by liquefaction of samples in dithiothreitol as described previously (5, 6). Dilutions were made and plated on selective media, and the plate with the countable number of colonies was used for enumeration and qualitative description of colonies. Isolates of *P. aeruginosa* with distinctive colonial morphologies on the basis of texture (mucoid or nonmucoid, rough or smooth edge), colony size, and pigmentation were designated unique and were enumerated separately and subcultured for MIC determination. Identification was made by conventional methods (13).

Antimicrobial agents and susceptibility tests. Susceptibility testing was performed by a broth microdilution method with media and under test conditions in accordance with the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS) (23). The test was conducted with commercially prepared dry panels and a semiautomated system according to the manufacturer's instructions. (Sensititre; AccuMed, Westlake, Ohio). Each phenotypically distinct *P. aeruginosa* isolate was tested against the following seven antibiotics (concentration ranges tested): tobramycin (0.25 to 512 μg/ml), gentamicin (0.25 to 512 μg/ml), amikacin (0.5 to 64 μg/ml), ticarcillin (2 to 4,096 μg/ml), ceftazidime (1 to 2,048 μg/ml), aztreonam (2 to 512 μg/ml), and ciprofloxacin (0.5 to 4 μg/ml). The isolates were categorized as susceptible, intermediate, or resistant according to NCCLS guidelines (23).

Determination of aminoglycoside resistance mechanisms. Mechanisms of resistance to aminoglycosides in *P. aeruginosa* isolates were studied by two meth-

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TABLE 1. Antibiograms for 1,240 *P. aeruginosa* isolates for seven anti-pseudomonal antibiotics

Antibiotic class	Antibiotic	Susceptibility breakpoint (μg/ml)	MIC (μg/ml)			Percent[a]		
			Range	50%	90%	S	I	R
Aminoglycoside	Tobramycin	≤4	≤0.25–>512	1	8	89.0	5.6	5.4
	Amikacin	≤16	≤0.5–>64	8	64	77.7	9.2	13.1
	Gentamicin	≤4	≤0.25–>512	4	32	64.8	15.9	19.3
β-Lactam	Aztreonam	≤8	≤2–>512	≤2	32	80.0	8.1	11.9
	Ceftazidime	≤8	≤1–>2,048	2	32	83.3	5.6	11.1
	Ticarcillin	≤64	≤2–>4,096	16	128	83.2	0.1	16.7
Quinolone	Ciprofloxacin	≤1	≤0.5–>4	1	4	63.2	16.1	20.7

[a] S, susceptible; I, intermediate; R, resistant.

ods: aminoglycoside resistance profile (AGRP) and DNA hybridization for specific aminoglycoside-modifying enzymes. AGRP was determined by the Kirby-Bauer disk diffusion assay with a panel of 12 different aminoglycosides as described previously (26). The known enzymatic resistance mechanisms result in distinctive patterns of decreased susceptibility (smaller zone diameters). Resistance to all 12 aminoglycosides has been operationally defined as impermeability (16, 20). AGRP has been shown to be capable of discerning which inactivating enzymes are possibly involved. However, this technique alone may not be capable of sorting out separate components that play a role in resistance. Therefore, a second approach to the study of resistance mechanisms was used to detect the presence of genes that encode known aminoglycoside-modifying enzymes. Southern blot analysis was performed as described previously (16, 26) with the following radiolabeled DNA probes: AAC(2′)-Ia, AAC(3)-Ia, AAC(3)-Ib, AAC(3)-Va, AAC(3)-VI, AAC(6′)-Ia, AAC(6′)-Ib, AAC(6′)-Ic, AAC(6′)-If, AAC(6′)-Il, AAC(6′)-Im, AAC(6′)-In, AAC(6′)-IIb, ANT(2″)-Ia, ANT(4′)-I, ANT(4′)-II, APH(3′)-I, APH(3′)-II, APH(3′)-III, and APH(3′)-VI. If hybridization with this large array of probes was negative and if AGRP tests indicated resistance to all aminoglycosides, the impermeability mechanism was presumed to be the cause, as described previously (16), although the role of other enzymes cannot be ruled out. If hybridization was positive for a specific enzyme and if pan-resistance was observed by AGRP, then the isolate was labeled as having both mechanisms, even though expression or inactivation was not determined.

RESULTS

In vitro susceptibility of *P. aeruginosa* isolates. A total of 1,240 isolates of *P. aeruginosa* were recovered from 508 patients at 69 geographically diverse centers in 34 states in the United States. The range of MICs, the MIC at which 50% of isolates are inhibited (MIC_{50}), the MIC_{90}, and percent distribution (susceptible, intermediate, and resistant) for each agent are listed in Table 1. Tobramycin was the most active aminoglycoside, and of all agents tested, the highest percentage of isolates were susceptible to tobramycin. Overall, only 5.4% of the isolates were classified as resistant on the basis of NCCLS criteria. This is compared to resistance rates of 20.7% for ciprofloxacin, 19.3% for gentamicin, 13.1% for amikacin, 16.7% for ticarcillin, 11.9% for aztreonam, and 11.1% for ceftazidime.

Isolates that were resistant to other agents were examined for their susceptibilities to tobramycin (Table 2), and isolates resistant to tobramycin were examined for their susceptibilities to the other agents (Table 3). Although cross-resistance among the aminoglycosides was common, 68 to 84% of the isolates resistant to β-lactams and/or ciprofloxacin were susceptible to tobramycin. For 67 isolates tobramycin MICs were ≥16 μg/ml. Of these resistant isolates, approximately one-third were susceptible to ciprofloxacin. More than half of the tobramycin-resistant isolates were susceptible to the β-lactams, with susceptibility to aztreonam being the highest (Table 3).

Antibiotic resistance of mucoid and nonmucoid *P. aeruginosa* isolates. Of the 1,240 isolates, 710 (57%) had the mucoid phenotype, a common finding among isolates of *P. aeruginosa* from patients with CF. To assess the relationship between mucoid isolates and antibiotic resistance, we compared the MIC data for the mucoid and nonmucoid isolate subsets (Fig. 1). For tobramycin, the frequencies of resistance for the nonmucoid and mucoid isolates were 9.4% (50 of 530) and 2.4% (17 of 710), respectively. For all antibiotics, the mucoid isolates were more susceptible to antibiotics.

Aminoglycoside resistance mechanisms. Of the 67 tobramycin-resistant isolates, 56 were available for aminoglycoside resistance studies. Of those, 51 demonstrated pan-resistance by AGRP tests, suggesting impermeability resistance. However, 2 of those 51 isolates (3.6%) hybridized with DNA sequences from genes encoding aminoglycoside-modifying enzymes (Table 4). The remaining five isolates (8.9%) hybridized with enzyme probes. Overall, 49 (87.5%) demonstrated the absence of hybridization with probes for known aminoglycoside-modifying enzymes and were presumed to be impermeability resistant.

DISCUSSION

This study provides important data on antimicrobial susceptibility trends for a large collection of recent clinical isolates of *P. aeruginosa* from CF patients. Because of the geographic diversity of isolates, this surveillance study provides a representative sample of the current susceptibility trends in the United States.

Compared with six other antimicrobial agents, tobramycin demonstrated excellent activity against *P. aeruginosa* isolates from CF patients. We observed that the frequency of resistance to tobramycin was lower than those to other aminoglycosides, common antipseudomonal β-lactams, and ciprofloxacin.

Our results are similar to those previously reported by others and suggest that tobramycin MICs are not increasing over time for isolates of *P. aeruginosa* from CF patients (1–3, 7, 12).

TABLE 2. Tobramycin susceptibilities of isolates resistant to other antibiotics

Agent to which isolate was resistant	No. of isolates	Isolates susceptible to tobramycin	
		No.	Percent
Amikacin	162	54	33.3
Gentamicin	239	106	44.4
Aztreonam	147	109	74.1
Ceftazidime	138	94	68.1
Ticarcillin	207	152	73.4
Ciprofloxacin	257	217	84.4

TABLE 3. Susceptibilities of tobramycin-resistant isolates to other antibiotics

Antibiotic	Isolates susceptible	
	No.	Percent
Amikacin	10	14.9
Gentamicin	0	0.0
Aztreonam	44	65.7
Ceftazidime	34	50.7
Ticarcillin	38	56.7
Ciprofloxacin	25	37.3

TABLE 4. Proposed tobramycin resistance mechanisms in 56 *P. aeruginosa* isolates

Resistance mechanism	No. of isolates	Tobramycin MIC (μg/ml)
Impermeability[a]	49	16–>512
Impermeability + AAC (6′)-Ib	2	32, 512
ANT (2″)-Ia	3	8, 16, 32
AAC (6′)-Ib	2	16, 16

[a] Negative for modifying enzymes by hybridization.

Although previous studies examined smaller numbers of isolates, MIC_{50}s and MIC_{90}s of tobramycin that we report are comparable. For example, Arguedas et al. (2) evaluated the in vitro activities of 10 antimicrobial agents against 50 strains of *P. aeruginosa* from 26 centers and reported tobramycin MIC_{50}s and MIC_{90}s of 2 and 64 μg/ml, respectively. Baltch et al. (3) tested 29 isolates of *P. aeruginosa* from patients with CF and reported tobramycin MIC_{50}s and MIC_{90}s of 2 and 8 μg/ml, respectively. In an earlier study, Lester and Andreasen (18) evaluated 30 *P. aeruginosa* isolates and found tobramycin MIC_{50}s and MIC_{90}s of 3.1 and 12.5 μg/ml, respectively, with up to 80% of strains being susceptible.

In a large study, Ciofu et al. (7) evaluated the susceptibilities of *P. aeruginosa* isolates collected during four different time periods over 18 years. They reported tobramycin geometric mean MICs of 0.5, 0.9, 2.6, and 3.0 μg/ml for the years 1973, 1980, 1985, and 1991, respectively. Despite this increase in tobramycin MICs, the investigators found that 98% of isolates remained susceptible. Much more dramatic increases in the MICs of ceftazidime, carbenicillin, and piperacillin have occurred. Others found similar increases in the MICs of ciprofloxacin as well (10).

These studies indicate little change in susceptibility to tobramycin, despite its extensive use in CF patients. This is in sharp contrast to the literature regarding susceptibility trends seen with other antibiotics such as ceftazidime and ciprofloxacin, which show dramatic decreases in activity over time (7, 10).

The susceptibility patterns of isolates of *P. aeruginosa* from patients with CF do not seem to parallel those of isolates from other patient populations. In 1984, Gordts et al. (14) compared the activities of various antibiotics against *P. aeruginosa* isolates obtained from CF patients with those obtained from patients with other chronic infections. A higher incidence of tobramycin resistance was reported among isolates from non-CF patients compared to the incidence among isolates from CF patients, while the activities of other agents were comparable (14). This observation appears to be corroborated by the recent study of the activities of meropenem and six other agents against 1,182 clinical isolates of *P. aeruginosa* from multiple laboratories across North America. Iaconis et al. (17) found that 26% of their isolates from non-CF patients were resistant to tobramycin, whereas only 5.4% of the isolates from patients with CF that we examined were resistant to tobramycin. In contrast, their results for ceftazidime resistance were similar to ours (15.6 versus 14%).

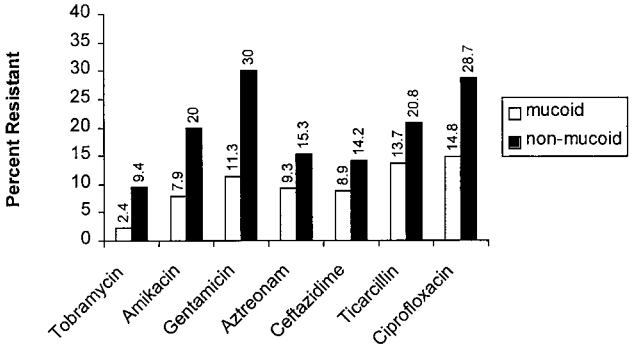

FIG. 1. Frequency of resistance among 710 mucoid and 530 nonmucoid isolates of *P. aeruginosa* recovered from CF patients. Breakpoints for resistance were as follows: tobramycin, ≥16 μg/ml; ceftazidime, ≥32 μg/ml; aztreonam, ≥32 μg/ml; amikacin, ≥64 μg/ml; ticarcillin, ≥128 μg/ml; gentamicin, ≥16 μg/ml; and ciprofloxacin, ≥4 μg/ml.

Because of frequent and prolonged antibiotic use in the CF patient population, the issue of multiple-antibiotic resistance is very important. The use of potentially synergistic antibiotic combinations has been recommended, and a clinically relevant definition of multiply antibiotic-resistant *P. aeruginosa* has been based on that recommendation (8). We evaluated our isolates for cross-resistance between tobramycin and other antipseudomonal agents. Tobramycin showed good activity against isolates resistant to other antibiotics: approximately 85% of ciprofloxacin-resistant and 70% of β-lactam-resistant isolates were susceptible to tobramycin. Tobramycin-resistant strains tended to be more resistant to other antibiotics, although 50 to 60% of those isolates remained susceptible to β-lactams.

The tobramycin-resistant isolates showed the expected cross-resistance to other aminoglycosides, but a significant proportion of these isolates were susceptible to ceftazidime, aztreonam, ticarcillin, and ciprofloxacin, suggesting that potential therapeutic options still exist for the rare patients from whom tobramycin-resistant isolates are recovered.

On the basis of NCCLS-recommended breakpoints for parenteral antibiotics, tobramycin resistance is defined as an MIC of ≥16 μg/ml. However, these isolates may respond if exposed to higher concentrations of drug. Recently, Saiman et al. (25) found that of 1,296 resistant *P. aeruginosa* isolates referred to their center for drug resistance and synergy studies, >90% were inhibited by 100 to 200 μg of tobramycin per ml. Drug delivery by aerosol provides a means of achieving such levels with clear clinical benefit (24). It has recently been shown that the mean concentration of tobramycin in the sputum of CF patients receiving inhaled tobramycin was 1,237 μg of sputum (6, 24). Although no new susceptibility breakpoint could be recommended for tobramycin delivered by the aerosol route, some patients colonized or infected with *P. aeruginosa* isolates for which tobramycin MICs are up to 128 μg/ml showed clinical improvement (6).

It has been suggested that isolates of *P. aeruginosa* with the mucoid phenotype are more resistant to antibiotics than nonmucoid isolates (4, 15). However, our data showed the opposite. For each of the drugs tested, a higher percentage of the

nonmucoid isolates than of the mucoid isolates were resistant. Similar observations have been noted previously for ciprofloxacin (10) and aminoglycosides (29). However, another study (27) found that the MIC_{90}s of ceftazidime, piperacillin, and amikacin were higher for mucoid isolates than for nonmucoid isolates, while no difference was seen for gentamicin, imipenem, and tobramycin. The significance and implications of this finding are unknown, but these results point out the need for further studies.

Aminoglycoside resistance in *P. aeruginosa* isolates from non-CF patients most often occurs by the acquisition of aminoglycoside-modifying enzymes (20, 21). In the current study, impermeability appeared to be the most prevalent mechanism of aminoglycoside resistance in *P. aeruginosa* isolates from CF patients. Only seven isolates were demonstrated to hybridize with DNA sequences from known genes for aminoglycoside-modifying enzymes. Therefore, resistance mechanisms in *P. aeruginosa* isolates from CF patients appear to be distinct from those in isolates from patients without CF. This finding is consistent with previous studies of isolates from patients with CF (16, 19, 25). However, this impermeability resistance has not been mechanistically defined, and novel enzymatic mechanisms, efflux, or target modification cannot be ruled out.

Despite years of use as antipseudomonal therapy, tobramycin appears to have maintained an excellent level of activity against *P. aeruginosa*. Our data suggest its continued value in the treatment of pulmonary infections in CF patients. In the nationwide survey described here, tobramycin was the most active of the antipseudomonal drugs tested, with the lowest overall rate of resistance caused apparently almost exclusively by nonenzymatic mechanisms. Although isolates may be resistant to more than one class of antibiotics, the majority of isolates that were resistant to ciprofloxacin or the β-lactam antibiotics remain susceptible to tobramycin.

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